Supporting Information

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SI Text

Western Blot Analysis. Promastigote lysates were prepared by suspending parasites rapidly in 1× Laemmli loading buffer and immediately boiling for 5 min. Samples (2×10^6 cell equivalents per lane) were subjected to SDS/PAGE and electrotransferred to nitrocellulose membranes (Hybond-ECL; Amersham Biosciences). Membranes were blocked with 5% nonfat dry milk in TBST (20 mM Tris-base, pH 7.6; 140 mM sodium chloride; and 0.05% Tween 20) for 1 h at room temperature. Protein loading was assessed with a rabbit anti-*L. major* histone H2A polyclonal antibody at 1:100,000 dilution. Polyclonal rabbit antibody anti-GFP (Abcam) was used at 1:2,500 dilution, and anti-FKBP12 (Affinity BioReagents) was used at 1:1,000 dilution; monoclonal antibody WIC79.3 (1) was used at 1:2,000 dilution. Goat anti-rabbit IgG HRP-conjugated secondary antibody (Jackson ImmunoResearch) was used at 1:10,000; anti-mouse IgG HRP-conjugated secondary antibody (Amersham Biosciences) was used at 1:5,000. ECL reactions were revealed with a PerkinElmer Life Sciences chemiluminescence kit. Quantitative Western blotting with WIC79.3 was performed with the Odissey Infrared Imaging System (LI-COR Biosciences) using the manufacturer's blocking buffer and goat anti-mouse IRDye 800CW secondary antibody (Li-Cor Biosciences). Quantitation was done with the Multi Gauge V3.0 software (Fujifilm).

ddFKBP Constructs. The oligonucleotides, templates, vectors, and constructs used or developed are described briefly below and with more detail in Table S2 and Table S3. All relevant regions of each construct were confirmed by sequencing and/or functional assays.

ORFs of interest were amplified by PCR and inserted into pGEM-T-Easy (Promega) by TA cloning according to the manufacturer's instructions. The YFP and ddYFP ORFs were then inserted into the *Leishmania* expression vector pIR1PHLEO (strain B4054) directly after digestion with BamHI, yielding pIR1PHLEO-ddYFP (strain B6145) and pIR1PHLEO-YFP (strain B6146).

To generate a general plasmid as an entry expression vector to fuse other genes of interest with the destabilizing domain, we amplified the ddFKBP segment with primers SMB3250 and SMB3251 and inserted the fragment in pGEM-T-Easy to generate pGEM-ddFKBP (strain B6177). The ddFKBP fragment was released by digestion with BclI plus BglII (by using a plasmid preparation obtained with a *dam-Escherichia coli* strain, SCS110) and ligated in the BamHI site of pIR1PHLEO, generating pIR1PHLEO-ddFKBP (strain B6182), which has convenient unique BamHI, SpeI, and XbaI sites into which ORFs can be inserted in frame (Fig. S5*C*). ORFs from the reporter luciferase (amplified from pGL3 Basic from Promega, which lacks the peroxisome-targeting C-terminal tripeptide) and several *L. major* genes (*FTL*: LmjF30.2600; *DHCH1*: LmjF26.0320; *DHFRTS*: LmjF06.0860; *GLF*: LmjF18.0200) were inserted into the BamHI site of B6182 to create pIR1PHLEO-ddFTL (strain B6247), pIR1PHLEO-ddDHCH (strain B6227), pIR1PHLEO-ddDHFRTS (strain B6332), and pIR1PHLEO-dd*GLF* (strain B6308) (Table S3).

Targeted Gene Replacement of *L. major GLF*. The 5′ *GLF* flanking region was amplified by PCR from *L. major* LV39 genomic DNA with primers SMB2188 and SMB2189, and the amplicon was digested by SalI plus SpeI and inserted into similarly digested pT7Blue (Novagen), generating plasmid B5327. The 3′ *GLF* flanking region was amplified by PCR with oligonucleotides SMB2190 and SMB2191, digested with SpeI plus KpnI, and inserted into similarly digested B5327, generating plasmid B5352. The *HYG* and *PAC* ORFs were then inserted between the internal SpeI/BamHI sites of B5352, yielding the desired *GLF* replacement constructs (B5356 and B5357, respectively). These constructs were digested with HindIII plus KpnI to yield the appropriate targeting fragment before transfection.

The first *GLF* allele was inactivated by using the *GLF*::*HYG* targeting construct. *L. major* LV39 clone 5 (Rho/SU/59/P) promastigotes were transfected by electroporation (2), and clonal transfectant lines were recovered after plating on 50 μg/mL hygromycin B. Multiple clonal lines showing the expected replacement by appropriate PCR tests were identified (*GLF*/*GLF*::Δ*HYG*). Several of these were infected into susceptible BALB/c mice at high inoculums (10^7). These showed lesion development typical of WT parasites, and after 1 month parasites were recovered. Clone H1 was chosen for targeting of the remaining *GLF* allele with the *GLF::PAC* replacement construct, and transfectants were recovered after plating on semisolid media containing 20 μg/mL puromycin and 50 μg/mL hygromycin B. Several clones were analyzed with regard to correct integration of the replacement cassette and the lack of the *GLF* ORF by PCR. Several homozygous replacements were obtained, and one of these was chosen for future work (Δ*GLF*::*HYG*/Δ*GLF*::*PAC* clone H1P1, referred to as *glf*$^-$ hereafter). This line was then transfected with pIR1PHLEO-dd*GLF*, yielding *glf*$^-$/+pIR1PHLEO-ddGLF, which expresses the ddGLF from the multicopy episomal pIR1PHLEO vector.

Generation of Knockin dd*GLF L. major*. To facilitate construction of ddFKBP knockin constructs, we created a generic ddFKBP knockin cassette with 3 key elements: a drug resistance marker (PHLEO), the DST gene intergenic region (DST IR), which has been extensively used to drive expression of downstream genes in *Leishmania* (3), and the ddFKBP sequence (strain B6323). We first digested pGEM-ddFKBP (strain B6177) with BclI plus BglII to release the ddFKBP fragment and ligated it in the BamHI site of pXGPHLEO (strain B3324), generating pXGPHLEO-ddFKBP (strain B6319). This plasmid was then used as template in PCR amplification with primers SMB2563 and SMB3251 to obtain the PHLEO-DST-ddFKBP fragment, which was cloned in pGEM-T-Easy, yielding pGEM-PHLEO-DST IR-dd (strain B6323; Fig. S6*A*). This is a generic knockin cassette; for any given gene of interest (GOI), it is necessary to insert the 5′ flanking sequence of the GOI ORF upstream of the PHLEO resistance marker (site A) and insert the ORF of the GOI in frame with the ddFKBP sequence (site B) (Fig. S6*A*).

To create the dd*GLF* knockin fragment, we released the 5′ flank of *GLF* used previously in the knockout cassette (strain B5327) with SalI plus SpeI digestion and inserted it into pGEM-PHLEO-DST IR-dd (strain B6323), which had been digested similarly, generating pGEM-5′UTR *GLF*-PHLEO-DST IR-dd (strain B6331). Finally, we inserted the ORF of *GLF* in the BamHI of B6331 in frame with the ddFKBP sequence, creating pGEM-kidd*GLF* (strain B6336). This targeting construct was released after digestion with NdeI plus SacII and was dephosphoryated before use.

The heterozygous *L. major* +/*glf*Δ*HYG* mutant clone H1 above was transfected with the knockin cassette by electroporation, and parasites were plated on semisolid M199 medium agar plates containing 50 μg/mL hygromycin, 10 μg/mL phleo-

mycin, and 2 μM FK506 to maintain ddGLF activity. Several colonies were picked and grown in liquid medium with 50 μg/mL hygromycin, 10 μg/mL phleomycin, and 1 μM FK506; correct integration of the cassette in the transfectants was verified by PCR. Parasites were cultivated in the presence of 1 μM FK506 at all times unless otherwise indicated.

1. Kelleher M, Bacic A, Handman E (1992) Identification of a macrophage-binding determinant on lipophosphoglycan from *Leishmania major* promastigotes. *Proc Natl Acad Sci USA* 89:6–10.
2. Robinson KA, Beverley SM (2003) Improvements in transfection efficiency and tests of RNA interference (RNAi) approaches in the protozoan parasite *Leishmania*. *Mol Biochem Parasitol* 128:217–228.
3. Ha DS, Schwarz JK, Turco SJ, Beverley SM (1996) Use of the green fluorescent protein as a marker in transfected *Leishmania*. *Mol Biochem Parasitol* 77:57–64.
4. Banaszynski LA, Chen LC, Maynard-Smith LA, Ooi AG, Wandless TJ (2006) A rapid, reversible and tunable method to regulate protein function in living cells using synthetic small molecules. *Cell* 126:995–1004.

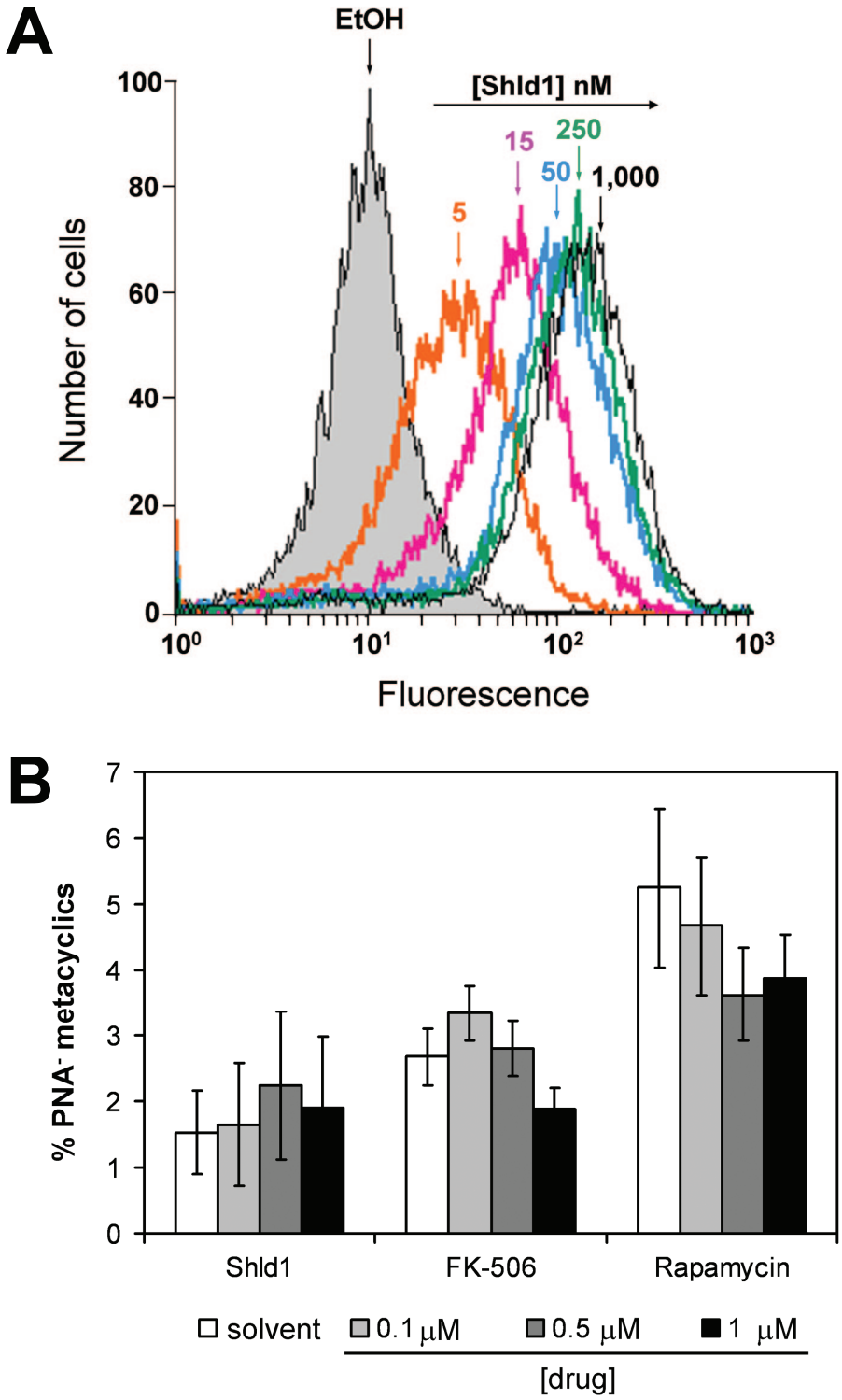

Fig. S1. Exploration of parameters relevant to the use of the ddFKBP regulatory system in *L. major*. (*A*) Tunability of the ddFKBP system. *L. major* transfectants expressing ddYFP (*SSU*::*IR1PHLEO-ddYFP*) were treated with different concentrations of Shld1 (5 nM, 15 nM, 50 nM, 250 nM, and 1 μM) for 24 h, and YFP fluorescence levels were measured by flow cytometry. Solvent (ethanol or EtOH)-treated cells were used as negative control. The figure shows strong, homogeneous expression increasing with increasing ligand concentration, the signature of tunability (4). (*B*) Effect of Shld1, FK506, and rapamycin on metacyclogenesis of *L. major*. To determine the effect of these compounds on metacyclogenesis, promastigotes were incubated with different concentrations of Shld1, FK506, or rapamycin until stationary day 2, and metacyclics were isolated by PNA methodology. Error bars represent SEM of 2 independent experiments performed in duplicate.

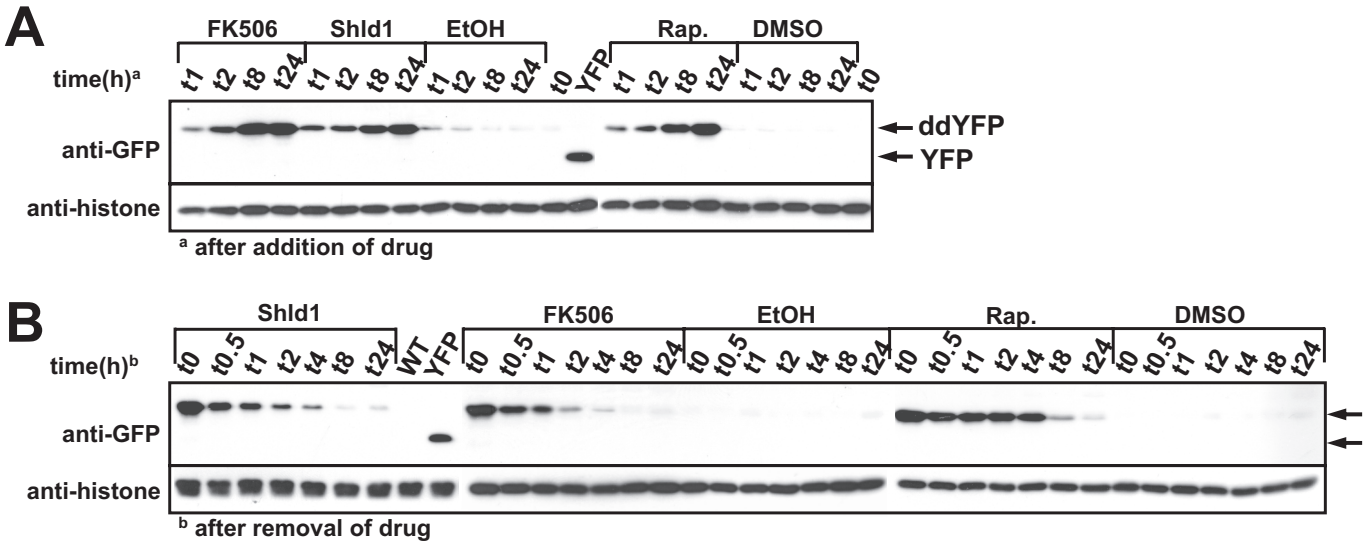

Fig. S2. Western blotting with anti-GFP antibodies confirms results obtained by flow cytometry (Fig. 3). (*A*) Stabilizing agents Shld1, FK506, or rapamycin were added to log phase *L. major* promastigote cultures, and samples were withdrawn at the time points indicated for Western blotting. (*B*) After a 24-h incubation with the indicated agents, parasites were washed and inoculated into media lacking drugs. Samples were collected at the indicated times and analyzed by Western blotting.

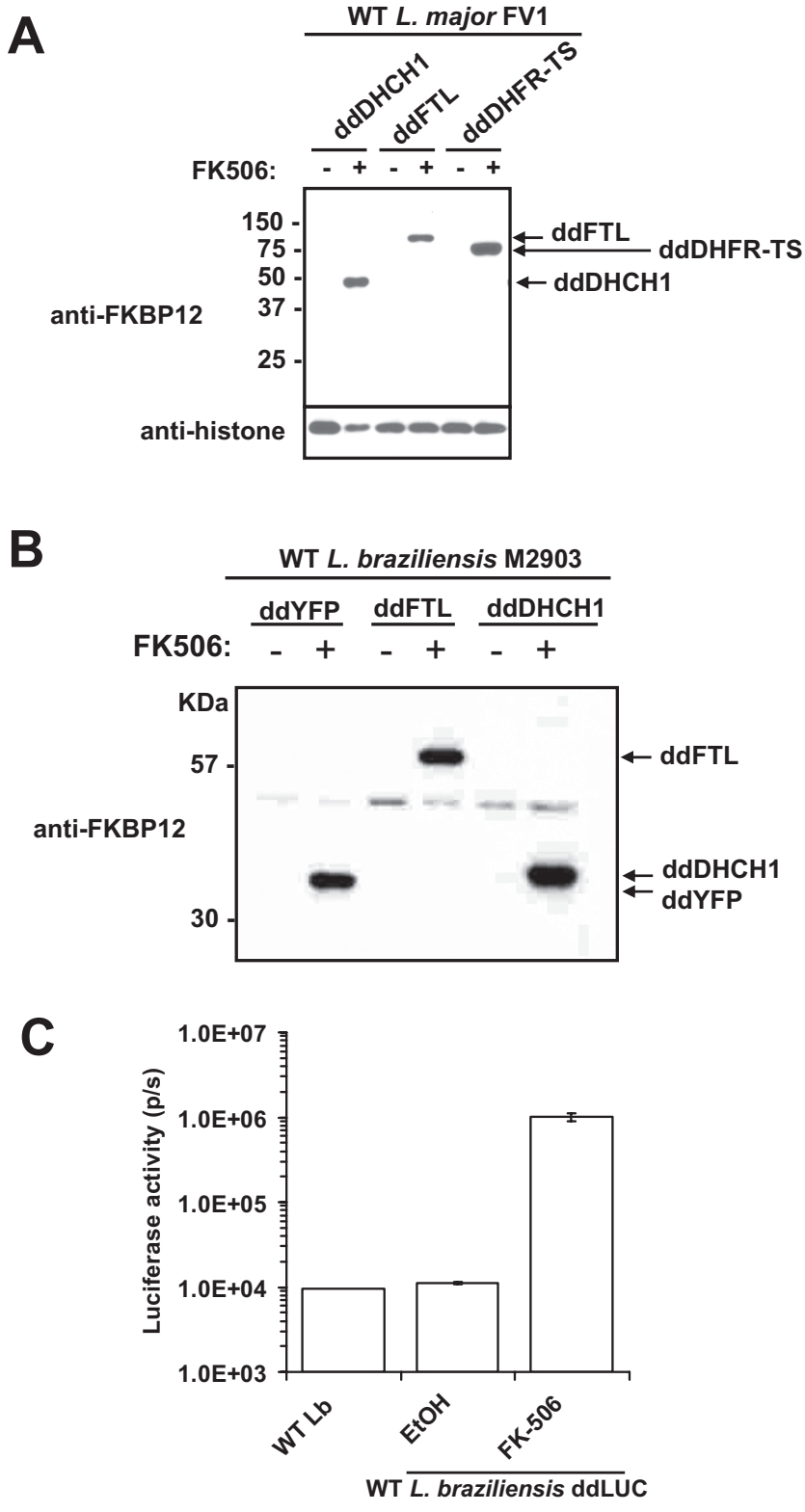

Fig. S3. Regulation of *Leishmania* proteins fused to the ddFKBP. (*A*) *L. major* transfectants: ddDHCH1, *SSU::IR1PHLEO-ddDHCH1*; ddFTL, *SSU::IR1PHLEO-ddFTL*; ddDHFR-TS, *SSU::IR1PHLEO-ddDHFR-TS*. Parasites were grown in logarithmic growth phase for 24 h in the presence/absence of 1 μM FK506 and were subjected to Western blotting with anti-FKBP antisera (*Upper*) or anti-histone H2B (loading control; *Lower*). The arrows mark the expected mobilities of the various dd fusion proteins. (*B*) Western blotting of *L. braziliensis* promastigote transfectants treated with FK506 as described in *A*. ddDHCH1, *SSU::IR1PHLEO-ddDHCH1*; ddFTL, *SSU::IR1PHLEO-ddFTL*; ddYFP, *SSU::IR1PHLEO-ddYFP*. (*C*) Luciferase activity of *L. braziliensis SSU::IR1PHLEO-ddLUC* transfectant promastigotes incubated 24 h with 1 μM FK506. We note that the luciferase cassette used here lacks the peroximal targeting signal, which in trypanosomatids targets proteins to the related organelle termed the glycosome.

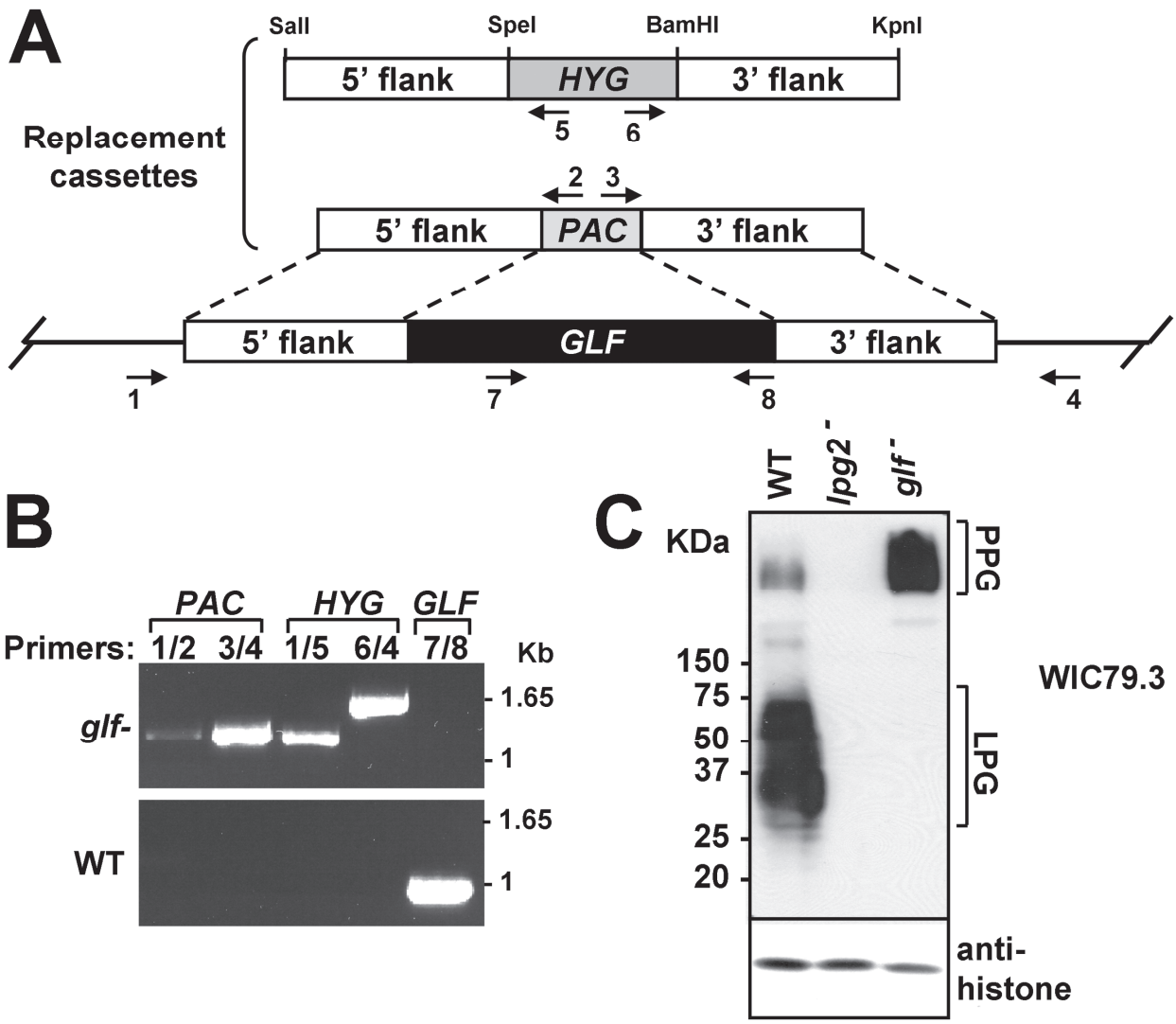

Fig. S4. Generation of *L. major glf*$^-$ null mutants. (*A*) Representation of the replacement targeting fragments used. (*B*) PCR analysis of a representative *glf*$^-$ mutant. Primers 1 and 2 together and primers 3 and 4 together establish the 5′ and 3′ sides of the PAC replacement; primers 1 and 5 together and 6 and 4 together establish the 5′ and 3′ sides of the HYG replacement; and primers 7 and 8 together confirm the presence or absence of the *GLF* ORF. Primers: 1, SMB3541; 2, SMB2889; 3, SMB2888; 4, SMB3542; 5, SMB2566; 6, SMB2565; 7, SMB2192; and 8, SMB3495 (Table S1). (*C*) LPG expression. (*Upper*) *L. major* phosphoglycans were visualized by Western blotting with WIC79.3 of whole-cell lysates of logarithmic-phase promastigotes (2×10^6 cell equivalents per lane). (*Lower*) shows a Western blot of these extracts with anti-*L. major* histone H2A as a loading control. Parasite lines were WT, a representative *glf*$^-$ mutant, and the *lpg2*$^-$ mutant described previously, which lacks all phosphoglycans [Spath GF, Garraway LA, Turco SJ, Beverley SM (2003) *Proc Natl Acad Sci USA* 100:9536–9541].

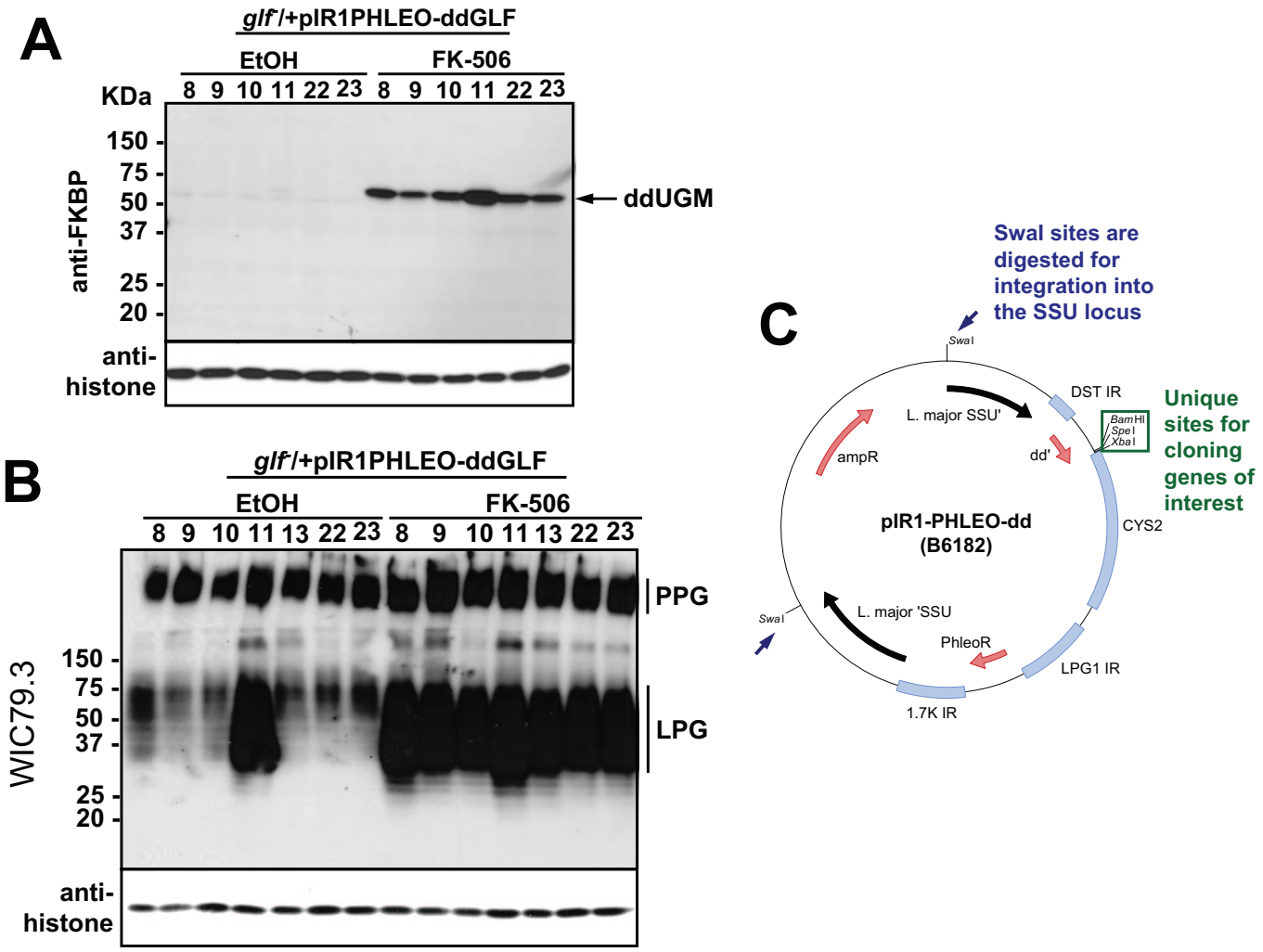

Fig. S5. Complementation of *glf*$^{-}$ knockout with episomal ddUGM expression. (*A* and *B*) Levels of ddUGM and LPG in 6 clonal *glf*$^{-}$*/+pIR1PHLEO-ddGLF* transfectants. Western blotting was performed with 3×10^6 cell equivalents per lane and using anti-FKBP12 (*A*) or WIC79.3 (*B*). The bars to the right of *B* mark reactivity associated with PPG or LPG, whereas the arrow in *A* marks the mobility of the ddUGM fusion protein. In each panel, the lower portion shows a Western blot with anti-*L. major* histone H2A polyclonal antibodies as a protein loading control. EtOH, cells treated with diluent (ethanol); FK506, cells treated with 1 μM FK506. Numbers 8, 9, 10, 11, 22, and 23 correspond to the identification numbers of the transfectants, which were chosen randomly. (*C*) Map of pIR1-Phleo-ddFKBP (B6182), a general *Leishmania* expression vector that can be used to tag GOIs with an N-terminal ddFKBP. This vector may be used to generate episomal transfectants using the intact plasmid, or it may be integrated by homologous recombination into the SSU rRNA locus after digestion with SwaI to expose homologous ends. Red arrows represent ORFs of genes coding for selectable markers (resistance to ampicillin and phleomycin) and the FKBP dd. Blue depicts intergenic regions that drive expression of the dd-tagged protein and phleomycin resistance drug marker. Black arrows represent fragments of SSU locus that are used for homologous recombination to generate integrated transfectants with high protein expression levels.

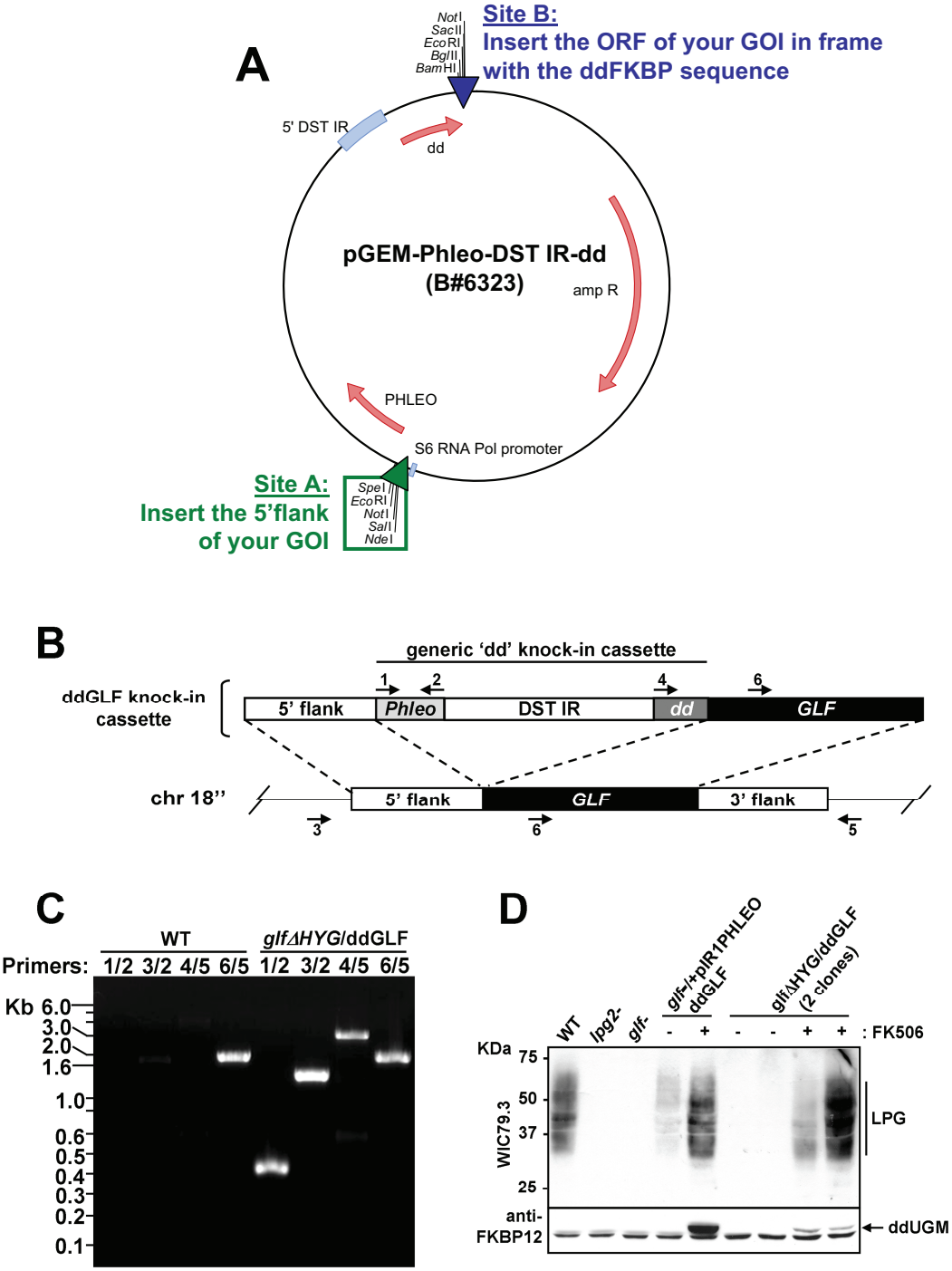

Fig. S6. Knockin strategy for in situ tagging of genes of interest (GOI) in *L. major* and its application to *GLF*. (*A*) Map of pGEM-Phleo-DST IR-ddFKBP (strain B6323), source of a useful general ddFKBP knockin cassette facilitating the tagging of genomic copies of GOIs with an N-terminal ddFKBP. To customize the cassette for a specific GOI, it is necessary to insert the 5′ flanking sequence of the GOI at the left position of the Phleo resistance marker (site A) and the ORF of the GOI in frame with the ddFKBP sequence (site B). (*B–D*) Generation of *ddGLF* knockin *L. major*. (*B*) Schematic representation of the knockin cassette and WT *GLF* locus. (*C*) PCR confirmation of successful integration of the *ddGLF* knockin cassette. Primers 1 and 2 together establish the presence of the PHLEO marker; primers 2 and 3 together and primers 4 and 5 together confirm the correct integration of the knockin cassette from the 5′ and 3′ sides, respectively; and primers 6 and 5 together confirm the presence of the GLF ORF. Primers: 1, SMB2563; 2, SMB2564; 3, SMB3541; 4, SMB3250; 5, SMB3542; and 6, SMB2186 (see Table S2 for sequences). (*D*) Expression of ddUGM fusion proteins (*Upper*) or LPG (*Lower*) by Western blotting with anti-FKBP antisera or monoclonal antibody WIC79.3, respectively. Parasite lines include WT, *glf*$^{-}$ and *lpg2*$^{-}$ controls, 1 representative *glf*$^{-}$/+pIR1PHLEO-ddGLF transfectant, and 2 representative ddUGM knockins. Samples are whole-cell lysates of logarithmic-phase promastigotes (3×10^6 cell equivalents per lane). A minus or plus sign indicates growth for 24 h in the presence of 1 μM FK506. A constant cross-reacting band with a mobility slightly below that of the ddUGM fusion protein is evident in the anti-FKBP12 blots and serves as a useful loading control; this band is seen in some but not all experiments (for example, compare *D* with Fig. S5*A*).

Table S1. Summary comparing the effects of Shld1, FK506, and rapamycin on the growth and expression of a ddYFP reporter in *SSU::IR1PHLEO-ddYFP* transfectant *L. major* promastigotes

Compound	EC_{50} half-maximum effective concentration, nM	EC_{100} maximum effective concentration, μM	Time for full induction, h (ON)	Time to decay 50%, h (OFF)	IC_{50} growth inhibition, μM
Shld1	10	0.25	8	0.5	8.4 $\pm$ 0.3*
FK506	200	1	8	0.5	7.7 $\pm$ 0.2
Rapamycin	60	1	8	1.25	4.9 $\pm$ 0.5

*The highest concentration of Shld1 was 1 μM because of limited availability and expense.

Table S2. Sequences of oligonucleotides

Primer no.	Sequence (5′ to 3′)
Primers used in destabilizing domain constructs	
SMB3225	GGATCC GCCACCATGGGAG
SMB3226	GGATCCCCACCATGGTGAGCAAGGGCGAGGAG
SMB3227	GGATCCGAGTTAGTCGAGTGCGTAGTCTGGTAC
SMB3250	TGATCACCACCATGGGAGTGCAGG
SMB3251	AGATCTGGATCCTTCCGGTTTTAGAAGCTCCACATC
SMB3543	AGATCTATGGAAGACGCCAAAAACAT
SMB3130	TTAGATCTTTACACGGCGATCTTTCCGC
SMB3573	GGATCCATGGCCACCCGGAAGCTGCA
SMB3574	GGATCCTTACGAAAGACCCACAATCC
SMB3575	GGATCCATGCCGTCTGCTCAGATCAT
SMB3576	GGATCCCTATGATACGCCCAACGCAG
SMB3494	GGATCCACCATGtacccatacgatgttccagattacgctAGCGCTGACAAGGTGGTCATAATC
SMB2180	GCATGCGGATCCTACGAGGCCGTCGACGACCATGTGCA
SMB3464	GGATCCATGTCCAGGGCAGCTGCGAGGTT
SMB3465	GGATCCCTATACGGCCATCTCCATCTTGA1
Primers used to construct the gene replacement knockout cassette for *L. major GLF*	
SMB2188	ATAGGCGTCGACGATGAGCGCAGGGGACGAGCA
SMB2189	ACTGACTAGTGATGGATTTGCTGCGTGTGCCTGCGT
SMB2190	ATTATACTAGTGCGGCCGCGGATCCAAGGTCGGCAGCCATGGACGA
SMB2191	GGGGAAGGTACCGCGGCTACATGCAGATCGTC
Primers used to construct the knockin cassette for *L. major GLF*	
SMB2563	GGTAACGGTGCGGGCTGACGCCACCATGGCCAAGTTGACCAGTGC
Primers used to confirm integration of *GLF* gene replacement knockout and dd knockin cassettes	
SMB3541	TGTTACGGTGATGCGCTAAG
SMB3542	CTCGTCCCCATTTCATCAAG
SMB2888	ACGTCGAGTGCCCGAAGGAC
SMB2889	ACCGTGGGCTTGTACTCGG
SMB2565	AATACGAGGTCGCCAACATC
SMB2566	GAAAGCACGAGATTCTTCGC
SMB2192	CCGCTGTGAACGTGGAGCGCATCA
SMB3495	GGATCCCGAGGCCGTCGACGACCATG
SMB2564	CGAGATCCCACGTAAGGTGCTCAGTCCTGCTCCTCGGCCACGAAG
SMB 2186	CGCGTAACCAAGGTGAACCCGATCACGAAGA

Underlining indicates restriction sites. Boldface indicates Kozak consensus sequence. Lowercase, HA tag.

Table S3. Constructs used in this work

Gene	Template	Oligos used for PCR	pGEM-T-easy intermediate	Leishmania expression construct
YFP	pBMN-FKBP(L106P)-YFP-HA(4)	SMB3226 and SMB3227	pGEM-YFP (strain B6143)	pIR1PHLEO-YFP (strain B6146)
ddYFP	pBMN-FKBP(L106P)-YFP-HA (4)	SMB3225 and SMB322	pGEM-ddYFP (strain B6142	pIR1PHLEO-ddYFP (strain B6145)
ddLUC	pGL3 Basic (Promega)	SMB3543 and SMB3130	pGEM-LUC (strain B6298)	pIR1PHLEO-ddLUC (strain B6299)
ddDHCH1	L. major FV1 genomic DNA	SMB3575 and SMB3576	pGEM-DHCH1 (strain B6353)	pIR1PHLEO-ddDHCH1 (strain B6227)
ddFTL	L. major FV1 genomic DNA	SMB3573 and SMB3574	pGEM-FTL (strain B6246)	pIR1-PHLEO-ddFTL (strain B6247)
ddDHFR-TS	pXG-LmDHFR-TS (B6098) (provided by Angela Li and Tim Vickers)	Plasmid was digested with BamHI for subcloning into pIR1PHLEO-ddFKBP (B6182)	none	pIR1PHLEO-ddDHFR-TS (strain B6332)
ddGLF	*L. major* FV1 genomic DNA	SMB3494 and SMB2180	pGEM-HA-GLF (strain B6307)	pIR1PHLEO-ddGLF (strain B6308)